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Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.)

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Abstract

The objective of this study was to evaluate the effects of hypoxia, elevated ammonia levels and crowding on the physiological and cellular stress responses of juvenile Nile tilapia *Oreochromis niloticus* (L.). The experiment was designed to be representative of prevalent harvest practices often encountered with this species. The levels of innate and inducible heat shock protein 70 (Hsp70) were measured in blood (packed cells), brain, liver, muscle and head kidney tissues of tilapia exposed to hypoxic stress (~ 4.9–0.3 mg l^{-1} dissolved oxygen), reduced water levels which tripled the fish/unit of water and an increase in ammonia levels from below detection limits on Day 1 to less than 0.5 mg l^{-1} at the end of 48 h. A highly significant increase in Hsp70 (***$P < 0.0001$) was detected in packed blood cells, brain, and muscle tissues in test fish compared to control treatments, but no differences were observed among treatments in liver (*$P = 0.93$) and head kidney (*$P = 0.24$) tissues. A highly significant increase (***$P < 0.0001$) was detected in serum glucose levels due to hypoxia treatments, and a statistically significant difference was detected among serum cortisol levels (*$P < 0.05$). The Hsp70 results reported here suggest that a physiological response as well as an inducible cellular stress response occurs in this species subjected to these stressful conditions, and that the cellular responses are tissue-specific and have not been observed in other teleosts species.

Keywords: Heat shock protein; Hsp70; Cortisol; Glucose; Nile tilapia; *Oreochromis niloticus*; Hypoxia

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1. Introduction

Many intensive fish culture systems are subjected to a lack of dissolved oxygen in the water due to high fish density and feeding practices, algal blooms and elevated temperatures which reduce the availability of oxygen. This lack of oxygen can induce both acute and chronic stress responses in fish (Pickering, 1981; Barton and Peter, 1982; Sumpter et al., 1986; Barton and Iwama, 1991). Typical metabolic adjustments made during hypoxic stress are to maintain oxygen supplies to critical organs, reduce energy metabolism, increase the energy supply by shifting to anaerobic glycolysis and expressing stress-related proteins (Hochachka and Somero, 1984; Dunn and Hochachka, 1986). It is important to understand the stress responses of fish under intense production pressure, particularly in response to changes in dissolved oxygen levels.

Heat shock proteins (Hsp's) also known as stress proteins consists of a family of molecules that play a pivotal role in the cellular stress response (Iwama et al., 1999; Basu et al., 2002). Studies have shown that they act as molecular chaperones associated with protein folding and assembly (Hartl, 1996; Pickard, 2002). These highly conserved cellular proteins are constitutive and inducible and have been demonstrated to protect cells when exposed to stress by modulating the recovery of aggregated and misfolded proteins (Morimoto et al., 1994; Hartl, 1996). Synthesis of Hsp increases in response to heat shock (Iwama et al., 1998) and to a variety of stressors including hyperosmolarity (Oehler et al., 1998), ischemia (Sanders et al., 1995; Mestril et al., 1994; Parsell and Lindquist, 1993), as well as superoxide radicals that are also formed during hypoxia and reoxygenation (Polla et al., 1998).

Hsp's are distinguished by their molecular weight and the most extensively characterized of the heat shock proteins is a 70 kDa protein (Hsp70).

Hsp70 is required for glucocorticoid receptor assembly (Hutchinson et al., 1994) and forms a heterocomplex with Hsp90 to facilitate the folding of the hormone binding domain (HBD) of receptors into the correct conformation. The ability of the hormone to control the HBD–chaperone interaction is thought to be the earliest event in the molecular pathway of steroid hormone action (Pratt and Toft, 1997). Hsp70 is also induced in many types of mammalian cells to provide increased resistance to ischemic and hypoxic injury (Ferriero et al., 1990; Iwaki et al., 1993; Patel et al., 1995; Turman et al., 1997; Oehler et al., 1998; Polla et al., 1998). Stress protein in mammalian cardiac tissue increases to almost three times baseline levels in response to brief ischemia (Marber et al., 1995). However, in two species of fish, rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) cells or tissues did not have elevated Hsp70 levels in response to hypoxia (Currie and Tufts, 1997; Gamperl et al., 1998; Airaksinen et al., 1998; Currie et al., 1999). No studies have previously reported an increase in Hsp70 in the blood cells of fish subjected to a stressor. Van Waarde et al. (1990) and Van Ginneken et al. (1995), reported a flexible metabolic depression occurring in anoxic fish muscles of Mozambique tilapia (*O. mossambicus*). These fish survive under anoxic/hypoxic conditions by lowering oxygen consumption without augmenting the activation of anaerobic metabolism. They are capable of reducing their oxygen consumption by 90% under hypoxic conditions compared to that under normal environmental conditions (Van Ginneken et al., 1995). The tolerance of extreme environmental conditions is presumed to be a physiological

adaptation phenomenon. However, the precise role of stress proteins in this species remains unclear. Many intensive fish culture systems are subjected to a lack of dissolved oxygen in the water due to high fish density, feeding practices, algal blooms and elevated temperatures which contribute to reduce the availability of oxygen. Lack of oxygen can induce both acute and chronic stress responses in fish (Pickering, 1981; Barton and Peter, 1982; Sumpter et al., 1986; Barton and Iwama, 1991). Typically, metabolic adjustments made during hypoxic stress are effected to maintain oxygen supplies to critical organ's, reduce energy metabolism, increase the energy supply by shifting to anaerobic glycolysis and expressing stress-related proteins (Hochachka and Somero, 1984; Dunn and Hochachka, 1986). It is important to understand the stress responses of fish under intensive production pressure, particularly in response to fluctuations in dissolved oxygen levels.

In this study, we examined the cellular stress response of Nile tilapia (*Oreochromis niloticus* L.), a warm water fish tolerant of crowding, high ammonia levels and low levels of dissolved oxygen (Stickney, 2000) by measuring the constitutive and inducible Hsp70 protein in packed blood cells, brain, muscle, liver and head kidney tissues. Serum glucose and cortisol were also measured as indicators of the physiological stress response.

2. Materials and methods

2.1. Fish

Juvenile mixed sex Nile tilapia (12.0 ± 0.02 cm standard error (SE), 35.2 ± 1.08 g (SE) were bred and maintained at the U.S. Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory at Auburn, AL. One week prior to the experiment, 10 fish were randomly placed into each of six, 15-l glass aquaria to a final density of 23.5 ± 0.02 kg/m^3, and supplied with flow through heated, dechlorinated, municipal water at a rate of 1.0–1.5 l min^{-1}. Three aquaria were assigned to test and three to control treatments using a random number table. Total ammonia was below detection limits and pH 7–7.5. Temperature was maintained at 27 ± 1 °C during the acclimation phase and at 24.4 ± 3.8 °C during the test. The water was continuously aerated in the controls during both the acclimation phase and during the test. The fish were fed 11.0 g of a commercial diet once daily. Photoperiod was a 12:12-h light/dark cycle.

2.2. Experimental protocols

On Day 1, water quality parameters were recorded, then aeration and incoming water was eliminated in the test treatment aquaria. Dissolved oxygen, temperature, ammonia and pH were monitored daily. Day 2, the water volume was reduced to 5 l, resulting in a density of 70.4 ± 2.16 kg/m^3 in all aquaria. Fish were not fed on Day 3 to avoid transient increases in cortisol levels (Bry, 1982).

On Day 3 (48 h after initiation of the experiment), 10 fish were removed from the aquaria individually and a blood sample taken from the caudal vein. The fish was killed by medullar section, weight and length were measured, and 100–500 mg of tissue

removed for determination of Hsp70 levels. All samples in the replication were taken within 10 min. Tissues were excised and placed in 2 ml screw cap cryovials (Corning, Corning, NY), and immediately immersed in liquid nitrogen. Storage was at − 80 °C until processing.

Blood samples were placed into 1 ml Microtainer© blood collection tubes containing a gel separation layer (Becton Dickinson, Franklin Lakes, NJ). Samples were immediately transported to the laboratory, centrifuged for 5 min at 16,000 rpm after clotting, the serum separated from the cells, then immersed in liquid nitrogen and maintained at − 80 °C until analysis.

2.2.1. Hsp70 extraction

A modification of the method of Forsyth et al. (1997) was used for extraction. One hundred milligrams of frozen tissue was removed from storage and placed in a scintillation vial with 2 ml ice-cold extraction buffer (1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF) in phosphate buffered saline (PBS).

Samples were homogenized (VirTis, Cyclone Vitishear) for 2 min on dry ice and sonicated (VirTis, Virsonic) for 5 min total time on dry ice using 30-s pulsations. Samples were centrifuged for 5 min at 14,000 rpm. The supernatant was aliquoted, refrozen on dry ice and the samples maintained frozen at − 80 °C until analysis.

Packed blood cells were weighed and diluted 1:20 wt/v with extraction buffer (described previously), sonicated for 1 min and refrozen on dry ice until analysis.

2.2.2. ELISA

Equal volumes of tissue extract was mixed with 0.1N NaOH and held for 15 min at room temperature. One hundred microliters of this extract was added to 100 μl coating buffer (sodium azide 3.07 mM, sodium bicarbonate 348.76 mM, sodium carbonate 150 mM, pH 9.5) and maintained at 4 °C overnight. After binding of proteins overnight at 4 °C, plates were washed three times with PBS-T (PBS, 0.05% v/v Tween-20), drained and blocked for 1 h with nonfat dry milk blocking buffer (NFDM, 5 g nonfat dry milk, 125 μl Tween 20, 75 μl heat-inactivated horse serum, 250 ml PBS). The plates were washed three times with PBS-T between each subsequent assay step. The primary antibody was monoclonal antibody Hsp70 (MAHsp70) (StressGen, Victoria, BC). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (GAM) (Sigma, St. Louis, MO). The antibodies were added to diluting layer (0.5% bovine serum albumin, 0.3% horse serum, 0.1% Tween 20, 0.01% thimersol, 0.005% phenol red, in PBS). MAHsp70 was added to the plates, 100 μl/well, at a 1:5000 dilution for 1 h at room temperature. GAM was added to the plates, 100 μl/well, at a 1:2500 dilution for 1 h at room temperature. The plates were incubated for 1 h at room temperature with 100 μl/well of substrate *o*-phenylenediamine dihydrochloride (OPD) (Sigma). The reaction was stopped with 0.3 M sulfuric acid and the absorbance was measured at 450 nm with a microplate reader (Dynatech MR 5000™). The total amounts of protein bound to each well were assumed to be equal and to be representative of the proteins present in the lysate. A standard curve was constructed by using a positive-control derived from rainbow trout gonad heat shocked cell lysate

Table 1
Dissolved oxygen and temperature profiles of control and hypoxia treatments for Nile tilapia

	Day 1		Day 2		Day 3	
	Control	Hypoxia	Control	Hypoxia	Control	Hypoxia
Temperature (°C)	24.9 ± 0.1	27.0 ± 1.7	25.3 ± 0.1	24.6 ± 1.2	24.9 ± 0.1	22.2 ± 2.4
Dissolved oxygen (mg l^{-1})	4.68 ± 0.6	4.91 ± 0.5	4.66 ± 0.8	3.7 ± 0.1	4.78 ± 0.3	0.3 ± 0.1
% Saturation (est.)	56	62	62	44	56	5

Temperature and dissolved oxygen values are the mean ± SE. $N = 10$.

(StressGen). Positive-control lysate was serially diluted in coating buffer and the standard curve plotted was linear over the assay range. Chemical induction of Hsp70 using Nile tilapia liver tissue, stressed in vitro with 50 μM sodium arsenite at 27 °C was unsuccessful (Forsyth et al., 1997).

2.2.3. ELISA

Proteins (20 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using 4–20% gels (BioRad, Hercules, CA) at 200 V for 30 min at 27 °C. The proteins were stained using GelCode Blue (Pierce Biotechnology, Rockford, IL).

2.2.4. Western blot

For Western blots, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using 4–20% gels (BioRad) at 200 V for 30 min at 27 °C. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad) at 30 V for 24 h in Towbin (Towbin et al., 1979) transfer buffer (48 mM tris, 39 mM glycine, 20% v/v methanol, pH 8.3) and a wet transfer apparatus (BioRad, Mini-Protean II Cell™). After blocking of the PVDF blots, Hsp-70 was detected by incubating for 1 h with anti-MAHsp70 antibody (StressGen), 1 h with goat anti-mouse antibody (Sigma), and 5–15 min with substrate (BioRad, Opti-4CN™ Substrate Kit). Gels and blots were visualized using BioRad Quantity 1™ equipment and software. Protein levels were determined using a commercially available bicinchoninic acid procedure (Pierce Biotechnology).

2.2.5. Glucose determination

Blood glucose levels in serum were determined using an enzymatic, glucose oxidase kit (Sigma, catalog number 510-A).

Table 2
Hsp70 extracts from Nile tilapia exposed to normal oxygenation and hypoxia

	Blood	Brain	Muscle	Liver	Head kidney
Control	0.312 ± 0.020 A	0.128 ± 0.009 A	0.166 ± 0.034 A	0.292 ± 0.018 A	0.198 ± 0.008 A
Hypoxia	0.488 ± 0.072 B	0.209 ± 0.019 B	0.383 ± 0.037 B	0.294 ± 0.016 A	0.184 ± 0.008 A

Values are optical density units normalized to 0.1 g tissue extracted. Values are the mean ± standard error. Means followed by the same letter are not significantly different at ***$P < 0.0001$. $N = 10$.

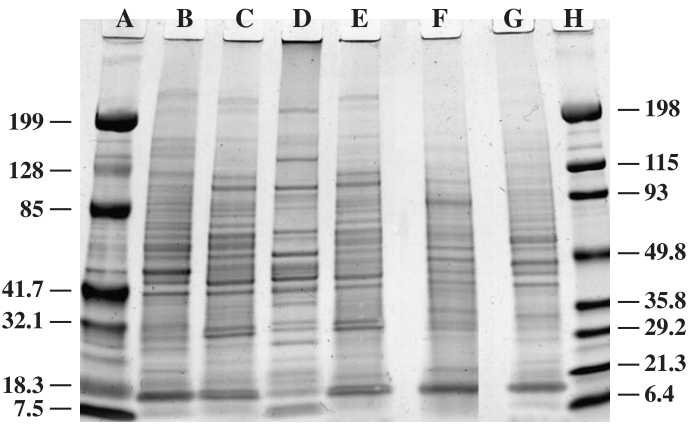

Fig. 1. SDS-PAGE of Hsp70 extracted from Nile tilapia tissues. All sample lanes contain 20 μg of protein. (Lane A) Kaleidoscope broad range molecular weight standard (kDa); (lane B) brain extract-hypoxia; (lane C) brain extract control; (lane D) muscle extract-hypoxia; (lane E) muscle extract control; (lane F) liver extract-hypoxia; (lane G) head kidney extract-hypoxia; (lane H) broad range standard (kDa).

2.2.6. Cortisol determination

Cortisol levels in serum were determined using a solid phase enzyme immunoassay kit (DRG Instruments, Martburg, Germany).

3. Statistics

The experimental design was completely randomized with two treatments and three replicates per treatment. Each replicate consisted of 10 fish as subsamples. Hsp70, glucose and cortisol values were analyzed by a one-way analysis of variance (ANOVA), followed by Duncan's means comparison ($P>0.05$). The completely randomized design of the experiments eliminates the effect of replication in the statistics. There was no

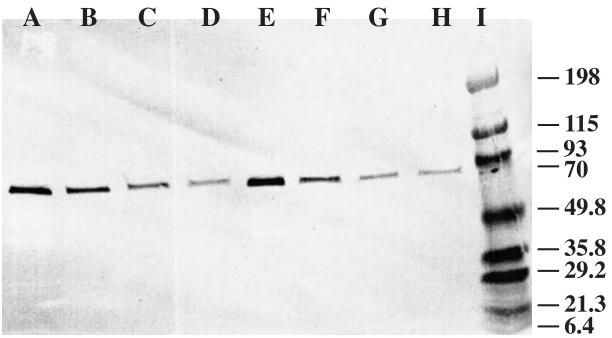

Fig. 2. Western blot of Hsp70 from tilapia brain extract. Lanes A–D are serial dilutions of control extract, lanes E–H are serial dilutions of hypoxia extract. A broad range molecular weight standard (kDa) spiked with Hsp70 is in lane I. Lanes A and E contain 20 μg of protein.

Table 3
Glucose and cortisol levels in juvenile Nile tilapia exposed to hypoxic stress

	Glucose (mmol l^{-1})	Cortisol (ng ml^{-1})
Control	99.1 ± 7.6 A	54.7 ± 42.0 A
Hypoxia	217.5 ± 9.2 B	88.4 ± 86.1 B

Values are the mean ± the standard error. Means followed by the same letter are not significantly different at ***$P < 0.0001$ for glucose and *$P < 0.05$ for cortisol. $N = 10$.

effect due to the time taken to collect the blood samples following treatments ($P > 0.05$) among fish for Hsp70 expression in any tissue, cortisol or glucose values determined by covariate analysis.

4. Results

Dissolved oxygen gradually decreased during the first few hours, then more rapidly when the water volume was decreased on Day 2 (Table 1). No distress was observed at the beginning of Day 2 prior to reducing the water volume. The fish became increasingly agitated and the observed respiratory frequency increased as the dissolved oxygen decreased. At the time of sampling the test fish (48 h), the fish were lethargic, respiratory frequency remained high and the fish were gasping at the air–water interface. Ammonia in the test aquaria remained below 0.5 mg l^{-1} and pH was less than 8 in all tanks after 48 h. Ammonia in the control tanks was below detection limits and pH = 7. The decrease in temperature observed in the hypoxia tanks is due to the lack of flowing heated city water that allowed the tanks to acclimate to room temperature.

A highly significant difference in Hsp70 (*$P = 0.0001$) was found between hypoxic and normal treatments in blood, brain and in muscle; however, no significant changes were determined between liver and head kidney samples (Table 2). Hypoxic and normal tissue extracts ran by SDS-PAGE are presented in Fig. 1 and a Western blot of serially diluted control and hypoxic brain extracts in Fig. 2. The gels support the ELISA data and increases in the Hsp70 are visible in the brain and muscle extracts compared to the controls. Although additional protein bands appear to be affected by the hypoxia treatment, no attempt was made to identify them. The Western blot serial dilutions confirm the presence of Hsp70. The antibody used does not differentiate between the constitutive and inducible forms of Hsp70, the proteins were not separated electrophoretically and the band intensities represent the total of the Hsp70 components. A highly significant increase in glucose (***$P < 0.0001$) and a significant increase in cortisol levels (*$P < 0.05$) was observed in the stressed fish (Table 3).

5. Discussion

Induction of Hsp70 has not previously been reported in teleosts due to hypoxic or anoxic conditions. In their normal range, tilapia are exposed to extremes of temperature and water quality and are able to utilize oxygen from the air–water interface to survive

low oxygen conditions. At the tissue level, cultured pancreatic islet cells of Nile tilapia survive at a lower oxygen tension than mammalian islet cells (Wright et al., 1989), and oxygen consumption decreases in the entire body under hypoxic conditions in many species of tilapia (Mohamed, 1981; Yamomoto, 1989; Van Ginneken et al., 1995).

We have not observed Hsp70 induction between treatments due to high total ammonia concentrations (>1.0 mg l^{-1}), holding the fish in a net for 5 min, short hypoxic periods (less than 15 min duration), chemical induction in liver tissue using sodium arsenite, decreasing the water temperature by >20 °C, or in response to crowded conditions (unpublished data). The crowding, decrease in temperature and the elevation of ammonia in the treatment tanks are probably not responsible for the increase in Hsp levels, although the influence of a combination of factors responsible cannot be concluded from this study.

In this study we have observed the induction of Hsp70 in the blood, brain and the muscle of Nile tilapia after 48 h of exposure to a low oxygen environment. The tissue-specific changes observed remain somewhat enigmatic. The induction of Hsp70 due to hypoxia is similar to the results observed in mammals, although occurring at a relatively slower rate. Mammalian cells will respond rapidly by synthesizing Hsp's when exposed to hypoxia and/or ischemia (Mestril et al., 1994; Mestril and Dillman, 1995). Benjamin et al. (1990) has presented evidence that hypoxia and heat shock induce expression of the Hsp70 gene by similar if not identical mechanisms in mammalian cells, although this may not hold to be true in teleosts.

In fish, the type and duration of exposure to a stressor, the tissue type and species specific heat shock factors may govern Hsp70 induction. Gamperl et al. (1998) found constitutive levels of Hsp70 in the myocardium of chinook salmon, but observed no induction after 6 h at dissolved oxygen concentrations of 3 mg l^{-1}. Currie and Tufts (1997) found constitutively expressed levels of Hsp70 in rainbow trout red blood cells (whole blood); however, inducible levels were not found after 2 h exposure to a nitrogen atmosphere. A later experiment demonstrated that hypoxia alone did not induce Hsp transcription in rainbow trout red blood cells (whole blood) despite a significant depression in ATP (Currie et al., 1999). This study suggests that a reduction in the cellular energy status may limit Hsp induction. In a hypoxia-tolerant, tropical species this may not be true.

In fish inhabiting low-oxygen water areas, the hemoglobin–oxygen affinity is higher than that of fish in other regions (Ninkinmaa, 2001; Powers et al., 1979). In teleosts, multiple hemoglobin types are present and they have different oxygen affinities (Weber and Jensen, 1988). Ninkinmaa (2001) suggests that one possible way of adapting to hypoxic environments would be by shifting the ratio of high-affinity and low-affinity hemoglobins via de novo synthesis. Changes in hemoglobin patterns have been reported due to hypoxic conditions (Marinsky et al., 1990); however, the significance of this change is unclear. The combination of cellular swelling, increase in cellular pH, decrease in guanosine and adenosine triphosphate may all be factors influencing the synthesis of Hsp70 in the blood cells under hypoxic conditions.

The mechanisms regulating heat shock protein gene expression have not been extensively studied in fish, and no reports are available for tilapia. Currie and Tufts (1997) reported that Hsp70 in rainbow trout may be regulated primarily at the transcriptional level. A heat shock factor (HSF) 1-like factor was reported to be involved in the induction of Hsp70 mRNA in rainbow trout by Airaksinen et al. (1998). Recently,

Rabergh et al. (2000) reported the cloning of this transcription factor in zebrafish (*Danio rerio*) as well as a fragment of HSF from bluegill sunfish (*Lepomis macrochirus*). Sequence homology of the fragment of cloned bluegill sunfish HSF suggests that fish may possess several distinct HSFs or that different species have divergent HSFs (Rabergh et al., 2000). The sequence homology was closer in the cloned bluegill HSF to human, mouse and chicken HSF than it was to the zebrafish gene (Rabergh et al., 2000). Dietz and Somero (1993) have reported a wide range of variability in threshold Hsp induction temperatures and levels among marine species and in constitutive levels among different tissues within a species when heat shock alone was used. They inferred that it was not a result of acclimation temperatures, but may be reflecting the influences of other factors including phylogeny. Basu et al. (2002) has suggested that it is possible that differences in a species' thermal history over recent or evolutionary time may have an influence on genetic or environmental regulation of Hsp induction. In the Antarctic fish *Trematomus bernacchii*, Hoffman et al. (2000) did not observe Hsp induction under chemical or heat treatments, however, mRNA levels changed drastically. The apparent lack of Hsp70 induction in the liver and head kidney could be deceiving. Ishibashi et al. (2002) observed the liver ATP content was reduced to only 5.5% of its initial value by low oxygen exposure and suggests that the liver adapts to hypoxic conditions by reducing its energy status. In Mozambique tilapia, goldfish (*Carrasius auratus*) and common carp (*Cyprinus carpio*), the survival method under anoxic and/or hypoxic conditions is activated by a decrease in oxygen consumption without complete activation of anaerobic metabolism (Hochachka, 1968). The mechanism that initiates the heat shock response is unknown; however, the slight decrease in liver ATP may not be sufficient to initiate the response.

The elevated Hsp70 levels in muscle and brain tissues may be related to a decrease in the available energy present in those tissues. It is possible that the ATP decreased in the tissues to a level which initiated the response.

Blood glucose and cortisol levels were significantly higher in the hypoxia treatments. Hypoxia is known to increase the levels of catecholamines, activating glycogenolysis and glyconeogenesis with a net result of increasing plasma glucose levels (Wright et al., 1989).

Although corticosteroids can be activated by hypoxia and stimulate the production of glucose, several authors have demonstrated that cortisol can also inhibit Hsp expression (Vijayan et al., 1997). Basu et al. (2001) has shown that cortisol may mediate Hsp70 levels following times of physiological stress. Further studies will be needed to assess the mechanisms that induce the Hsp response in specific tissues under metabolic stress.

6. Conclusion

We report here significantly increased levels of inducible Hsp70 in blood, brain and muscle tissues of Nile tilapia exposed to hypoxia. The physiological adaptations that allow Nile tilapia to survive poor water quality also appear to be accompanied by tissue-specific Hsp70 induction. Although increases in Hsp70 were observed in several tissues, further research will be needed to determine the significance of these findings. The biochemical and physiological signals that induce Hsp70 synthesis remain unknown, and appear to be tissue- as well as species-dependent.

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